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Natural Cyclopeptides as Leads for Novel Pesticides: Tentoxin and Destruxin*

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Abstract: Difficulties in synthesis make natural cyclopeptides challenging targets for chemists. Our interest focused on two natural toxic cyclopeptide series produced by pathogenic fungi: tentoxin, [cyclo-(N-MeAla[1]-Leu[2]-N-MeΔ^zPhe[3]-Gly[4])] and the destruxins [cyclo-(Pro[1]-Ile[2]-N-MeVal[3]-N-MeAla[4]-β-Ala[5]-HA[6])]. The total syntheses of these two bioactive series were optimised, and several analogues were designed and synthesised to establish structure–activity relationships. The importance of synthetic analogues in the identification of molecular targets and the explanation of mechanisms of action was demonstrated. Such systematic investigations can determine the crucial features responsible for the activity of the natural compound and help the design of more powerful or more selective products. © 1998 SCI.

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Key words: cyclopeptide; cyclodepsipeptide; tentoxin; F_0F_1 H^+-ATPase; herbicide; destruxin; insecticide

Abbreviations: These follow the recommendations of the IUPAC-IUB Commission on Biological Nomenclature as given in *Eur. J. Biochem.* **138** (1984), 9–37. Additional abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Boc, *tert*-butyloxycarbonyl; BOP, (1*H*-1,2,3-benzotriazol-1-yloxy)-tris(dimethylamino)-phosphonium hexafluorophosphate; BroP, bromo-tris(dimethylamino)-phosphonium hexafluorophosphate; DCC, dicyclocarbodiimide; DMAP, 4-dimethylaminopyridine; DDQ, dicyanodichloroquinone; DPPA, diphenylphosphoryl azide; DTT, dithiothreitol; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Lac, lactic acid; Hpp, 2-hydroxy-3-phenylpropionic acid; Hpy, 2-hydroxy 4-propynoic acid; Piv-Cl, pivaloyl chloride; TFA, trifluoroacetic acid; TMS, trimethylsilyl; Z, benzyloxycarbonyl.

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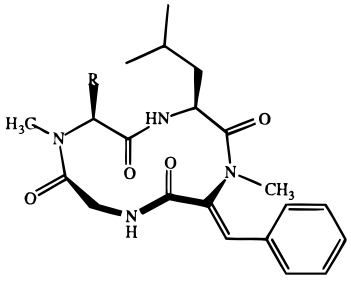
1 INTRODUCTION

Interest in cyclic peptides dates back half a century to the discovery that the antibiotic gramicidin S is a cyclic decapeptide.[1] Since then numerous natural cyclic peptides acting as antibiotics and toxins have been found. A variety of biological studies have suggested that cyclic structures may exhibit improved metabolic stabilities, increased potencies, better receptor selectivities and more controlled bioavailabilities than linear peptides. Further, the constrained geometries of cyclic peptides favour conformational investigations and can help in locking one key structural element. Among these molecules, the cyclotetrapeptide tentoxin [cyclo-(N-MeAla^1-Leu^2-N-MeΔ^zPhe^3-Gly^4)] (Fig. 1) and the cyclodepsipeptide destruxins [cyclo-(Pro^1-Ile^2-N-MeVal^3-N-MeAla^4-β-Ala^5-HA^6)] (Fig. 2) are of particular interest, hence this study of their structural, biochemical and physiological features.

These cyclopeptides are produced by pathogenic fungi. Destruxins were first isolated by Kodaira[2] from the entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorokin, and recently from the entomogenous fungus *Aschersonia sp.*[3] Other destruxins, such as destruxin B, are produced by phytopathogenic fungi like *Ophiosphaerella herpotricha*[4] and *Alternaria brassicae* (Berk.) Sacc.[5] Tentoxin is produced by a phytopathogenic fungus of the same family, *Alternaria alternata* (Fr.) Keissler (*A. tenuis* Nees).[6]

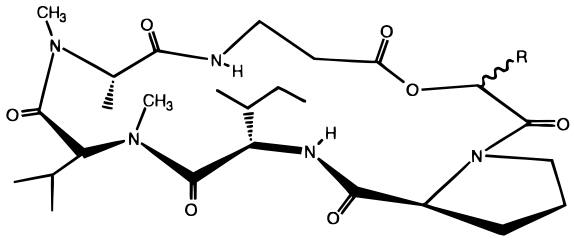
These toxins have different pathogenic effects. Destruxins isolated from *M. anisopliae* have an insecticidal activity,[7–9] decreasing in the order E, A and B.[10,11] Some insecticidal properties could be related to the inhibition of immune reactions.[12–15] Destruxins from entomogenous fungi also possess antitumour[16–18] and antiviral[19,20] properties. Those of the destruxins extracted from *A. brassicae*, particularly destruxin B, have shown phytotoxic properties mainly directed against Brassicae and other crucifers.[21] Tentoxin exhibits phytotoxicity by inducing chlorosis of many plants.[6,22,23]

Molecular targets of these toxins have been identified in few cases. Examples are the voltage-dependent calcium channel that destruxin B activated in insect muscle, leading to a paralysis of the host.[24] Experiments with lepidopteran cell lines showed that destruxin E induced a calcium influx, without evidence for a direct interaction with calcium channels.[25] At the same time, a rapid transient phosphorylation of a 250 kD protein was induced by destruxin E, suggesting the activation of a protein kinase.[25] But the most clearly characterised molecular targets of both fungal cyclic peptides belong



R	Compound	
CH_3	Tentoxin	1
CH_2-OBn	MeSer(Bn)^1-Tentoxin	2
CH_2-OH	MeSer^1-Tentoxin	3
CH_2-CO_2tBu	MeGlu(tBu)^1-Tentoxin	4
CH_2-CO_2H	MeGlu^1-Tentoxin	5

Fig. 1. Structures of tentoxin analogues.



R	Compound		R	Compound	
	Destruxin E	6	CH_2-C_6H_5	(D) Hpp-6 destruxin E	12a
CH_3	(D)Lac-6 destruxin E	7	CH_2-C_6H_5	(L) Hpp-6 destruxin E	12b
CH_2-$CH(CH_3)$-CH_2OH	Destruxin C	8	CH_2—C≡CH	(D) Hpy-6 destruxin E	13a
CH_2-$CH(CH_3)$-CO_2H	Destruxin D	9	CH_2—C≡CH	(L) Hpy-6 destruxin E	13b
CH_2-CH=CH_2	Destruxin A	10	CH_2—C≡C—TMS	(D) Hpy(TMS)-6 destruxin E	14a
CH_2-$CH(CH_3)_2$	Destruxin B	11	CH_2—C≡C—TMS	(L) Hpy(TMS)-6 destruxin E	14b

Fig. 2. Structures of destruxin analogues.

to the family of proton ATPases. On the one hand, vacuolar H^+-ATPase has been shown[26] or indirectly suggested[27] to be inhibited by destruxin B and E respectively. On the other hand, chloroplast F-type H^+-ATPase is inhibited by tentoxin, which binds specifically on the extrinsic part CF_1 of the enzyme.[28] The effect of tentoxin is concentration-dependent: at low concentration (10^{-8} M) it is a powerful inhibitor, while above 10^{-5} M it stimulates the enzyme[29–31] *in vitro*. The latter effect is probably not relevant *in vivo*.

We have developed a general approach for the total synthesis and the chemical modification of these natural toxins. Often, the limiting step in small cyclopeptide synthesis is cyclisation. Intramolecular cyclisation is precluded by the rigidity of the linear precursor, due to the π character of the peptide bond. Different solutions have been suggested in the literature[32,33] but they are often unsatisfactory. The ring-closure site has a determining influence on the yield of cyclisation. We have demonstrated, from theoretical calculations performed on five cyclotetrapeptides using the GenMol program,[34] that the yield of the ring-closure reaction is strongly dependent on the energy barrier associated with the predicted transition state.[35] All the linear precursors of a cyclopeptide can be unfavourable for cyclisation, unless a structural feature such as an *N*-methylated residue, a proline or a D-amino acid is present in the peptide. These features are often found in natural peptides, and we have developed general methods of cyclisation adapted to these compounds, based on a reversible modification of the peptide backbone.[36]

It also turns out that each cyclopeptide synthesis is unique and strongly dependent on the nature of the residues and the sequence. According to our approach, it is necessary to define the best ring-closure site to optimize the cyclisation yield. In this report, we validate this approach throughout the synthesis of natural bioactive cyclopeptides tentoxin, destruxins and analogues. Results of biological studies on these newly designed molecules are also presented.

2 METHODS

2.1 Synthesis of tentoxin and derivatives

Structures of tentoxin and analogues are displayed in Fig. 1. In the synthesis of tentoxin, the choice of linear peptide precursor is limited. Dehydro amino acids are weakly reactive, thus N-MeΔ^zPhe cannot be at a terminus, and N-MeAla is not suitable for the C-terminal position because racemisation can occur.[37–39] Consequently, a sequence with Gly at the C-terminus seems the most rational possibility and allows the use of more drastic cyclisation conditions without any risk of racemisation. The same closure site was chosen in all syntheses.

All analogue precursors were synthesised following the same pathway, employing an azlactone as key intermediate,[40] which presents the advantage of introducing the variable residue at the last step (Fig. 3). Starting from the same tripeptide Leu-N-MeΔPhe-GlyOMe, obtained in homogeneous phase, we prepared a set of five compounds (Fig. 1). When the methylated amino acid was coupled as a Boc-protected version in the case of N-MeSer, the synthesis led directly to tentoxin. Using the BocN-MeSer(Bn) derivative in the last coupling step afforded another analogue which was more hydrophobic. For the Glu-analogue, we used a Z-protected N-MeGlu(*tert*Bu) and the cleavage of the *tert*-Bu ester was carried out after cyclisation. Whereas the Boc-cleavage with TFA was uncritical, the course of Z-deprotection had to be carefully followed by HPLC. Indeed, the Z-deprotection had to be quenched before the double-bond hydrogenation.

Cyclisation yields using DPPA as reagent were low, ranging from 5 to 20%. We have recently increased the yield of cyclisation of tentoxin from 19 to 50% (isolated compound) by using the PPA reagent (*n*-propane phosphonic acid anhydride) provided by Hoechst.

2.2 Synthesis of destruxins

The nomenclature and formulae of destruxins and analogues are depicted in Fig. 2. Amino acids are in the natural L configuration. Hydroxy acid HA possesses D

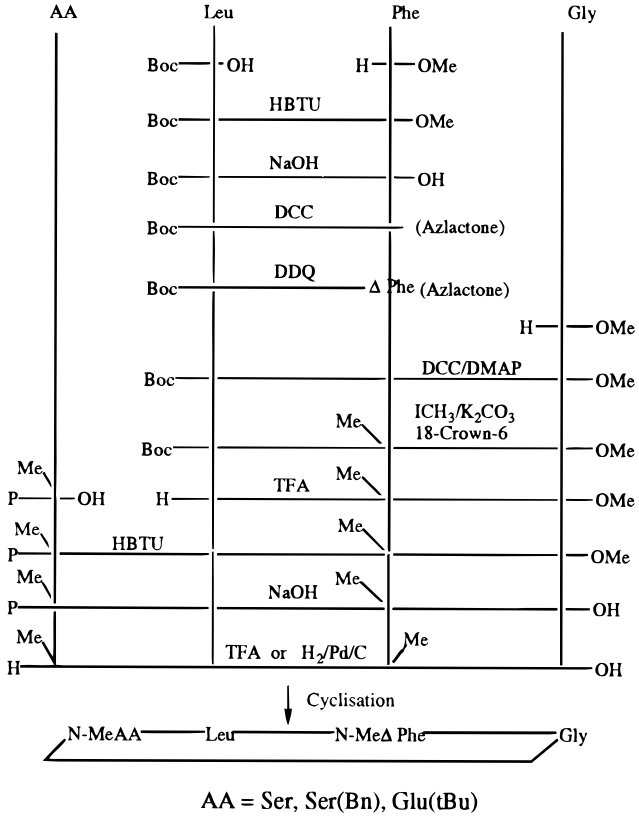


Fig. 3. Schematic pathway for synthesis of tentoxin precursors.

configuration in natural compounds and D or L in synthetic analogues.

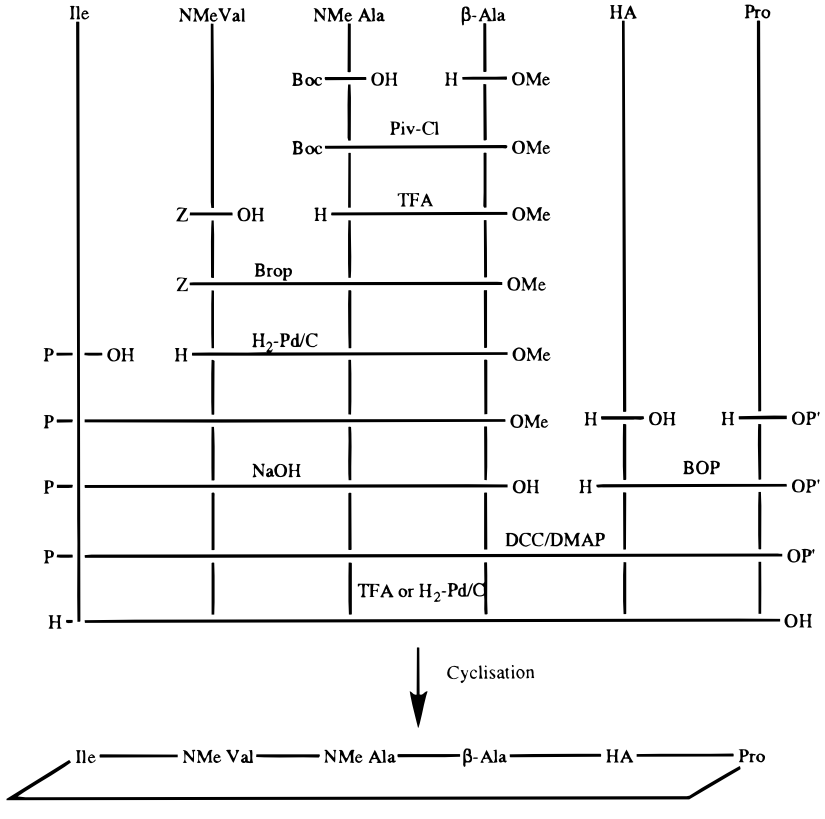
The synthesis of these compounds presents the difficulty of coupling two vicinal *N*-methyl amino acids. The chosen strategy allowed their early introduction in the synthesis. The tetrapeptide Ile-N-MeVal-N-MeAla-*β*-Ala was thus selected as the precursor. Cyclisation through the depsi bond, which is more difficult to form than an amide bond, is not suitable. In addition, proline at an *N*-terminus often gives rise to poor coupling yield. The only remaining possibility for the ring-closure site is thus the *C*-terminal proline, which adds the advantage of a decreased racemisation risk. This convergent strategy, described for the synthesis of Lac-6 destruxin E(**7**),[41] was applied to the preparation of peptidic analogues with different terminal protecting groups.[42] Indeed, we switched from the couple Boc/*tert*Bu to Z/Bn in order to avoid acidic conditions which were found to partially degrade the peptide. Compound **7** was obtained by cyclisation using DPPA as reagent in 30% yield. The fully peptidic compounds were prepared with cyclisation yields ranging from 60 to 78%. The same convergent synthesis was chosen to prepare the new cyclodepsipeptides **12, 13** and **14,** as depicted in Fig. 4.

Whereas the Hpps needed for the synthesis of the analogues **12a,b** are commercially available, we had to synthesise both the Hpy and Hpy(TMS) in both configurations for analogues **13a,b** and **14a,b** from (D) or (L) serine.[43] For the above reasons, the *Z*-strategy was preferred for the preparation of Hpp-6-destruxins E (**12**). However, the Boc/*tert*Bu protecting groups were used in the synthesis of Hpy-6-destruxins E(**13**) and of its protected analogues **14,** because the presence of the triple bond prohibits the hydrogenolysis conditions required in the Z/Bn deprotection step.

Starting from the hexapeptide Ile-N-MeVal-N-MeAla-*β*-Ala-(D)N-MeAla-Pro, we extensively studied the ring-closure reaction to optimise the yield. The best conditions were found to be BOP (10 eq.), DIEA (20 eq.) in chloroform.[43] The cyclodepsipeptides **12, 13** and **14** were obtained in yields of between 63 and 71% after purification by semi-preparative HPLC. All the cyclic depsipeptides have been characterised by high resolution mass spectroscopy and [^{1}H]NMR.[43]

2.3 Tentoxin: Assays on isolated CF_1

Isolation from spinach (*Spinacia oleracea* L.) leaves and purification of the CF_1 complex devoid of its inhibitory ε subunit (CF_1-ε) was carried out as previously described.[44] The enzyme was activated by incubation



P = Boc or Z
P' = tBu or Bn

Fig. 4. Schematic pathway for synthesis of destruxin analogues.

for 3 h at room temperature at a concentration of 80 μg ml^{-1} in a medium containing Tricine (20 mM) and DDT (3 mM), pH 8·0. For assays of ATP hydrolysis, the activated enzyme was diluted 40-fold in the reaction medium containing Tris-SO_4 (50 mM), $MgSO_4$ (0·18 mM), $KHCO_3$ (40 mM) pH 8·0. This medium was supplemented with toxins at indicated concentrations. After 5 min of incubation at 37°C, the reaction was triggered by adding ATP (1 mM). Portions were taken at different time intervals and analysed for nucleotides by HPLC, as described.[45] ADP concentration increased linearly with time, which allowed measurement of the rate of ATP hydrolysis.

2.4 Destruxin: Assays on insects

Assays of destruxin E and synthetic analogues were carried out according to the standard method previously developed for testing (D)Lac-6 destruxin E.[41] The compounds were injected to the larvae of the lepideptoran insect *Galleria mellonella* L. For the standard bioassays, each toxic solution was prepared by dissolution of 1 mg of product in 100 μl of acetone + methanol (1 + 1, by volume), and diluted with sodium chloride solution (6 g litre^{-1}, 900 μl). Larvae with a mean body weight of 125 mg were injected with 8 μl of solution. The toxicity of destruxins was tested on groups of 20 larvae, and evaluated by the paralysing and lethal effect of the treatment. Paralysis was determined by counting the fully paralysed larvae after a 30-min interval. The larval mortality was recorded at 2, 4, 7, 14 and 30 days and the cumulative percentage of mortality was calculated. For preliminary studies, we used only one determination. For a detailed comparison of the effects of destruxin E, four different concentrations corresponding to 2, 1, 0·25 and 0·06 mg ml^{-1} were injected, and the paralysis was noted after 1, 30 and 60 min. Three replicates were performed for each treatment.

3 RESULTS AND DISCUSSION

3.1 Design of toxin analogues

For a detailed investigation of the mechanism of action of tentoxin, as seen by its interaction with CF_1, it was crucial to synthesise the natural toxin in the best yield using a procedure that could be easily adjusted to the synthesis of new analogues. This was achieved with the use of an azlactone as key intermediate, and the choice of the most suitable linear precursor N-MeAA^1-Leu^2-N-MeΔ^zPhe^3-Gly^4. Furthermore, two-dimensional proton NMR structural studies of natural tentoxin[46] revealed the existence of four stable conformers slowly interconverting in aqueous solution (*cis-trans-cis-trans* conformation of the amide bonds sequence), all of them presenting a marked intramolecular stacking of Leu and Δ^zPhe side chains. NMR also indicated that the molecule tends to be organised in small aggregates to protect the hydrophobic moiety (Leu^2-N-MeΔ^zPhe^3) from the water molecules. On the basis of these results, together with previous reports, we speculated which of these chemical properties would account for the biological activity. In particular, the affinity for the binding site on CF_1 is likely to be related to the cyclic nature of the peptide associated with the alternate configuration *cis-trans-cis-trans*, and/or the planarity of the α,β dehydro amino acid, and/or the existence of a strongly hydrophobic pole in the molecule. Having regard to these features, the design of new active derivatives rationally turned to the substitution of side chains only, with *N*-methylated positions unchanged in order to maintain the conformational flexibility of the cyclic backbone. Because of the possible implication of the hydrophobic moiety Leu^2-N-MeΔ^zPhe^3 in the binding site, we chose to modify the side chain of residue 1 (N-MeAla). This choice allowed the preservation of the global molecular lipophilicity, while making possible the investigation of the effect induced by a local variation in hydrophobicity (N-MeAla^1 replaced by N-MeSer), or by the introduction of a charge (N-MeAla^1 replaced by N-MeGlu). In addition to the advantage of a useful chemical functionality on the side-chain, these modifications were consistent with the unified synthesis strategy described above.

The four tentoxin analogues synthesised (**2–5**) had a poor solubility in water. Their structural examination by two-dimensional proton NMR confirmed that they were self-associated in aqueous solution, similar to tentoxin. (André, F. Pinet, E. unpublished results). Our structural study also revealed that their cyclic backbones present the same conformations as the natural toxin. The conformational agreement with tentoxin was excellent even in the case of **3**,[45] which showed marked differences in biological activity (see below).

Regarding the design of destruxin derivatives, we initially chose the synthesis of a simple analogue, **7**, as a basis to develop a general methodology.[38] We then investigated different modifications of the structure to examine their effects on activity. We prepared a set of analogues in which the ester bond was replaced by an amide bond.[42] None of these compounds showed any insecticidal activity, thus attesting the requirement of the depside bond. We then focused our studies on the nature of the α-hydroxy acid, guided in the choice by some observations in natural destruxins: a hydrophilic function on the side-chain R, like an alcohol in **8** or a carboxylic acid in **9**, led to relatively inactive compounds. The *in-vivo* ring-opening of the destruxin E epoxide side-chain, affording a diol, is perceived as a way of detoxification.[47,48] On the other hand, a non-hydrophilic but electron-rich side-chain R improved the

activity. Indeed, the double-bond-containing **10** was more active than **11** in which the side-chain R is fully saturated. Furthermore, the epoxide-containing **6** was the most potent of the whole family. The lack of hydrophilic function and the presence of an unsaturated alkyl group were both therefore expected to contribute to good activity. These observations led us to design three destruxin analogues **12, 13** and **14** in which the α-hydroxy acid is respectively Hpp, Hpy and Hpy(TMS) in (D) or (L) configuration.

3.2 Activity of tentoxin derivatives

The effects of tentoxin derivatives (all modified on the N-MeAla position) on the ATPase activity of the isolated CF_1-ε are displayed in Table 1. All these compounds were efficient inhibitors, but at different concentrations. This is shown in Table 1 by the variation of IC_{50}, the concentration at which 50% inhibition was reached. Among the analogues checked, only **3** had an affinity for this site comparable to that of **1,** whereas **2, 4** and **5** exhibited lower affinities. So the introduction of a charge (**5**) or an increase of the steric volume (**2** and **4**) at the N-MeAla position makes the binding of the molecule more difficult but does not change its effect once it is bound.

At higher concentrations (>10 μM), tentoxin reactivates CF_1-ATPase, an effect that can be related to the binding of a second molecule to a low affinity site.[49] The maximal reactivation is always very high in the case of tentoxin (250% of the control in Table 1). N-MeSer-tentoxin at high concentration reactivated the ATPases poorly (only 30% of the control), while no reactivation all was observed with the other derivatives. The reason for the deficiency in reactivation is not the same for all the analogues. Competition experiments between tentoxin and these analogues (results not shown) have indicated indeed that **4** and **5** do not bind to the low-affinity site of CF_1. By contrast **2** and **3** were shown to bind to this site with about the same affinity as tentoxin, but the bound molecule was unable to reactivate the enzyme. The case of **3** is especially interesting, because a minor change of the molecule resulted in a single but important change in its properties.

TABLE 1
Effect of Tentoxin and Analogues on the Activity of CF_1-ATPase

Compound	IC_{50} (μM)	*Maximal reactivation* (%)
1	0·03	250
2	0·5	negligible
3	0·05	30
4	2	negligible
5	5	negligible

3.3 Activity of destruxin derivatives

The bioassays enabled an initial evaluation of the paralysing and insecticidal activities of these cyclodepsipeptides. We first demonstrated that the presence of the depside bond is essential for the activity. Further, the toxicity of the destruxin analogues for the larvae of the lepidopteran insect *G. mellonella* varied depending on the nature of the hydroxy acid. The results of the biological assays are summarised in Fig. 5.

Among the compounds with a (D)hydroxy acid, **12a** showed a low insecticidal activity, compared to the lethal activity of **7,** the mortality of the insects being 15 and 25% respectively. In contrast, **13a** exhibited a very good insecticidal effect. The final mortality rate was 65%, as also observed with **6.** Moreover, **12a** remained able to trigger a general and complete paralysis, already observed for **7.**[38]

For each hydroxy acid, the analogues containing an acid in (L) configuration were less active than those with a (D) configuration (data not shown). Thus, **12b** had not only the same poor lethal effect as the corresponding (D) derivative **12a,** but also no paralysing effect. In **13b** the capacity to trigger paralysis was also decreased as compared to the effect induced by the corresponding (D) derivative **13a.** The degree of paralysis 30 min after treatment decreased from 100 to 35%. In this case, the insecticidal effect was also reduced to a low level, the final mortality being only 30%.

The introduction of a trimethylsilyl group (**14a** and **14b**) resulted in a general loss of activity: **14b** completely lost the moderate toxicity of **13b.** Compound **14a** was also less toxic than the corresponding **13a** and destruxin E **6,** with paralysis of only 70% and a mortality close to that of the control.

After the initial assays had shown that **13a** was a potent insecticidal analogue, its paralysing and lethal

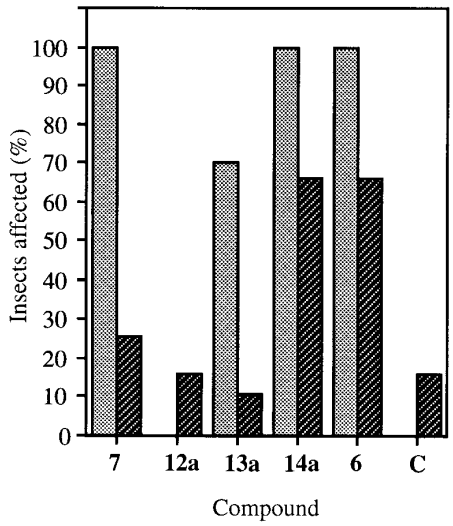


Fig. 5. (▒) Paralysis and (▨) mortality of *Galleria mellonella* caused by natural destruxin E (**6**) and destruxin analogues. C = control.

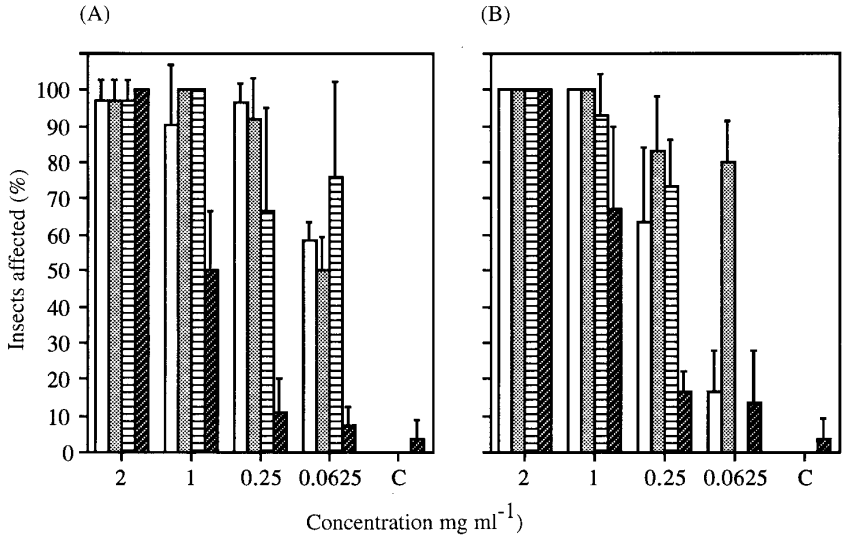


Fig. 6. Paralysis at (□) 1, (▨) 30 and (▤) 60 min, and (▨) mortality induced in *Galleria mellonella* by (A) natural destruxin E (**6**) and (B) analogue **13a.** C = control.

activity were compared in detail with the effects of destruxin E applied in identical conditions. The results obtained did not reveal a significant difference between the analogue and the most efficient of the natural destruxins (Figs 6a,b). Depending on the concentration of active products injected, changes occured in the percentage of insects exhibiting a full paralysis and in the speed of initiation of the paralysis and recovery. Thus the paralysis appeared more slowly at the lower concentrations with compound **13a.**

4 CONCLUSION

Based on two complementary examples, we illustrate the contribution of synthetic analogues of natural toxins in the development of new insecticides and herbicides. These structure–activity studies can help to identify some molecular features that are critical for biological activity and are expected to help in the design of more powerful and more selective products. The ability to synthesise the novel analogues is obviously a critical factor and these studies must rely on an efficient strategy of production of a large variety of analogues, which in these examples was dependent on the optimisation of the cyclisation process. Thus the destruxin analogue **13a** already appears as a potent analogue whose insecticidal and acaricidal properties are currently being investigated in more detail.

In addition, these new products also provide suitable tools both in the identification of the molecular target and in the elucidation of its mechanism of action. In this respect, biochemical and pharmacological studies need to be carried out to determine whether the mode of action at the cellular and subcellular level differs between the various destruxin analogues and natural destruxin E used as reference. Investigations of the nature and function of destruxin-binding proteins in insect cells, recently detected by *in-vitro* assays, are being actively pursued. Such research will have to take into account the fact that different effects are observed in lepidopterans and in other insects, such as locusts, which do not respond to destruxins by typical paralysis.

The tentoxin study is somewhat different, since one molecular target has already been clearly identified. In addition to the identification of other possible targets that could account for the chlorotic effect, future efforts can focus on the production of new derivatives and on the detailed study of their effects on chloroplast H^+-ATPase, including the location of the binding sites in the protein. This kind of approach is expected to contribute significantly to the understanding of the ATP synthesis mechanism.

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REFERENCES

1. Consden, R. J., Gordon, A. H., Martin, A. J. B. & Synge, R. L. M., Gramicidin S: the sequence of amino acid residues. *Biochem. J.*, **41** (1947) 596–602.
2. Kodaira, Y., Biochemical studies on the muscardine fungi in the silkwork, *Bombix mori. J. Fac. Textile Sericult., Shinshu Univ., Ser. E.*, **29** (1961) 1–68.
3. Krasnoff, S. B. & Gibson, D. M., New destruxins from the entomopathogenic fungus *Aschersonia sp. J. Nat. Prod.*, **59** (1996) 485–9.
4. Venkatasubbaiah, P., Tisserat, N. A. & Chilton, W. S., Metabolites of *Ophiosphaerella herpotricha*, a cause of

spring dead spot of bermudagrass. *Mycopathologia*, **128** (1994) 155–9.
5. Shivanna, K. R. & Sawhney, V. K., Pollen selection for Alternaria resistance in oilseed brassicas: responses of pollen grain and leaves to a toxin of *Alternaria brassicae*. *Theor. Applied Gen.*, **86** (1993) 339–44.
6. Fulton, N. D., Bollenbacher, K. & Templeton, G. E., A metabolite from *Alternaria tenuis* that inhibits chlorophyll production. *Phytopathology*, **55** (1965) 49–51.
7. Fargues, J., Robert, P. H. & Vey, A., Effet des destruxines A, B et E dans la pathogénvèse de *Metarhizium anisopliae* chez les larves de coléoptères Scarabaeidae. *Enthomophaga*, **30** (1985) 353–64.
8. Quiot, J. M., Vey, A. & Vago, C., Effects of mycotoxins on invertebrate cells *in vitro*. *Adv. Cell Cult.*, **4** (1985) 199–212.
9. Fargues, J., Robert, P. H., Vey, A. & Païs, M., Toxicité relative de la destruxine E pour le lépidoptère *Galleria mellonella* L., *C. R. Acad. Sci. Ser. 3*, **303** (1986) 83–6.
10. Gupta, S., Roberts, D. W. & Renwick, J. A. A., Preparative isolation of destruxins from *Metarhizium anisopliae* by high performance liquid chromatography. *J. Liq. Chromatogr.*, **12** (1989) 383–95.
11. Loutelier, C., Cherton, J.-C., Lange, C., Traris, M. & Vey, A., Studies on the dynamics of the production of destruxins by *Metarhizium anisoplae*. Direct high-performance liquid chromatographic and fast atom bombardment mass spectrometric analysis correlated with biological activity tests. *J. Chromatography A*, **738** (1996) 181–9.
12. Vey, A. & Quiot, J. M., Effet *in vitro* de substances toxiques produites par le champignon *Metarhizium anisopliae* (Metsch.) Sorok sur la réaction hémocytaire du coléoptère *Oryctes rhinoceros* L. *C. R. Acad. Sci. Ser. D*, **280** (1975) 931–4.
13. Vey, A., Quiot, J.-M., Vago, C. & Fargues, J., Effet immunodépresseur de toxines fongiques: inhibition de la réaction d'encapsulement multicellulaire par les destruxines. *C. R. Acad. Sci. Ser. 3*, **300** (1985) 647–51.
14. Huxham, I. M., Lackie, A. M. & McCorkindale, N. J., Inhibitory effects of cyclodepsipeptides, destruxins, from the fungus *Metarhizium anisopliae* on cellular immunity in insects. *J. Insect Physiol.*, **35** (1989) 97–105.
15. Cerenius, L., Thornqvist, P. O., Vey, A., Johansson, M. W. & Soderhall, K., The effect of the fungal toxin destruxin E on isolated crayfish hemocytes. *J. Insect Physiol.*, **36** (1990) 785–90.
16. Morel, E., Païs, M., Turpin, M. & Guyot, M., Cytotoxicity of cyclodepsipeptides on murine lymphocytes and on L1210 leukemia cells. *Biomed. Pharmacother.*, **37** (1983) 184–5.
17. Odier, F., Vago, P., Quiot, J.-M., Devauchelle, G. & Bureau, J.-P., Etude cytométrique des effets de la destruxine E sur des cellules leucémiques de souris. *C. R. Acad. Sci. Ser. 3*, **305** (1987) 575–8.
18. Odier, F., Vey, A. & Bureau, J.-P., *In-vitro* effect of fungal cyclodepsipeptides on leukemic cells: study of destruxins A, B and E. *Biol. Cell*, **74** (1992) 267–71.
19. Quiot, J. M., Vey, A., Vago, C. & Païs, M., Action antivirale d'une mycotoxine. Etude d'une toxine de l'hyphomycète *Metarhizium anisopliae* (Metsch.) Sorok en culture cellulaire. *C. R. Acad. Sci. Ser. D*, **291** (1980) 763–6.
20. Kopecky, J., Matha, V. & Jegorov, A., The inhibitory effect of destruxin A on the replication of arboviruses in *Hedes albopictus* C6/36 cell line. *Comp. Biochem. Physiol.*, **103C** (1992) 23–5.
21. Buchwaldt, L. & Green, H., Phytotoxicity of destruxin B and its possible role in the pathogenesis of Alternaria brassicae. *Plant Pathol.*, **41** (1992) 55–63.
22. Lax, A. R., Shepherd, H. S. & Edwards, J. V., Tentoxin, a chlorosis-inducing toxin from *Alternaria* as a potential herbicide. *Weed Technol.*, **2** (1988) 540–4.
23. Durbin, R. D. & Uchytil, T. F., A survey of plant insensitivity to tentoxin. *Phytopathology*, **67** (1977) 602–3.
24. Bradfisch, G. A. & Harmer, S. L., Omega-Conotoxin GVIA and nifedipine inhibit the depolarizing action of the fungal metabolite, destruxin B, on muscle from the tobacco budworm (*Heliothis virescens*). *Toxicon*, **28** (1991) 1249–54.
25. Dumas, C., Matha, V., Quiot, J.-M. & Vey, A., Effects of destruxins, cyclic depsipeptide mycotoxins, on calcium balance and phosphorylation of intracellular proteins in lepidopteran cell lines. *Comp. Biochem. Physiol.*, **114C** (1996) 213–19.
26. Muroi, M., Shiragami, N. & Takatsuki, A., Destruxin B, a specific and readily reversible inhibitor of vacuolar-type H^+-translocating ATPase. *Biochem. Biophys. Res. Commun.*, **205** (1994) 1358–65.
27. Naganuma, S., Kuzuya, N., Sakai, K., Hasumi, K. & Endo, A., Inhibition of the accumulation of lipid droplets in macrophage J774 by bafilomycin B1 and destruxin E. *Biochim. Biophys. Acta*, **1126** (1992) 41–8.
28. Steele, J. A., Uchytil, T. F., Durbin, R. D., Bhatnagar, P. & Rich, D. H., Chloroplast coupling factor 1: a species-specific receptor for tentoxin. *Proc. Natl Acad. Sci. USA*, **73** (1976) 2245–8.
29. Hu, N., Mills, D. A., Huchzermeyer, B. & Richter, M. L., Inhibition by tentoxin of cooperativity among nucleotide binding sites on chloroplast coupling factor 1. *J. Biol. Chem.*, **268** (1993) 8536–40.
30. Dahse, I., Pezennec, S., Girault, G., Berger, G., André, F. & Liebermann, B., The interaction of tentoxin with CF_1 and CF_1-ε isolated from spinach chloroplast. *J. Plant. Physiol.*, **143** (1994) 615–20.
31. Sigalat, C., Pitard, B. & Haraux, F., Proton coupling is preserved in membrane-bound chloroplast ATPase activated by high concentration of tentoxin. *FEBS Lett.*, **368** (1995) 253–6.
32. Spatola, A. F., Anwer, M. K., Rockwell, A. L. & Gierash, L. M., Compatibility of β- and γ-turn features with a peptide backbone modification: synthesis and conformational analysis of a model cyclic pseudopeptide. *J. Am. Chem. Soc.*, **108** (1986) 825–31.
33. Rovero, P., Quartara, L. & Fabbri, G., Synthesis of cyclic peptides on solid support. *Tetrahedron Lett.*, **32** (1991) 2639–42.
34. Pèpe, G. & Siri, D., GenMol. A fast program for molecular modeling. Application to the determination of the psychotonic or sedative effects of tricyclic antidepressant drugs. *Stud. Phys. Theor. Chem.*, **71** (1990) 93–101.
35. Cavelier, F., Pèpe, G., Verducci, J., Siri, D. & Jacquier, J., Prediction of the best linear precursor in the synthesis of cyclotetrapeptides by molecular mechanics calculations. *J. Am. Chem. Soc.*, **114** (1992) 8885–90.
36. Cavelier, F., Achmad, S., Verducci, J., Jacquier, J. & Pèpe, G., How to perform small peptide cyclisations. *J. Mol. Struct.*, **286** (1993) 125–30.
37. McDermott, J. & Benoiton, N., *N*-Methylamino Acids in Peptide Synthesis. III. Racemization during deprotection by saponification and acidolysis. *Can. J. Chem.*, **51** (1973) 2555–61.
38. Davies, J. & Karim Mohammed, A., Assessment of racemization in *N*-alkylated amino-acid derivatives during peptide coupling in a model dipeptide system. *J. C. S. Perkin I* (1981) 2982–90.
39. Cavelier-Frontin, F., Guendouz, F., Jacquier, R. & Verducci, J., Utilisation en synthèse peptidique de 4-

dialkylaminopyridines supportées sur polyméres. *Bull. Soc. Chim. Fr.*, **129** (1992) 463–7.
40. Cavelier, F. & Verducci, J., New synthesis of the cyclic tetrapeptide tentoxin employing an azlactone as key intermediate. *Tetrahedron Lett.*, **36** (1995) 4425–8.
41. Calmès, M., Cavelier-Frontin, F., Jacquier, R., Mercadier, J.-L., Sabil, S., Verducci, J., Quiot, J.-M. & Vey, A., Synthesis and biological activity of a destruxin analogue: D-Lac-6 destruxin E. *Int. J. Pept. Protein Res.*, **41** (1993) 528–35.
42. Cavelier, F., Jacquier, R., Mercadier, J.-L. & Verducci, J., Destruxin analogues: depsipeptidic bond replacement by amide bond. *Tetrahedron*, **52** (1996) 6173–86.
43. Cavelier, F., Verducci, J., Traris, M. & Vey, A., Destruxin analogues: variation of the α-hydroxy acid side chain. *J. Pept. Res.*, in press.
44. Berger, G., Girault, G., André, F. & Galmiche, J.-M., Application of HPLC to the purification of coupling factor CF_1 from spinach chloroplasts and of some of its subunits. *J. Liquid Chromatogr.*, **10** (1987) 1507–17.
45. Pinet, E., Cavelier, F., Verducci, J., Dubart, L., Girault, G. & André, F., Synthesis, structure and properties of MeSer1-Tentoxin, a new cyclic tetrapeptide which interacts specifically with chloroplast F_1 H^+-ATPase. Differentiation of inhibitory and stimulating effects. *Biochemistry*, **35** (1996) 12804–11.
46. Pinet, E., Neumann, J.-M., Dahse, I., Girault, G. & André, F., Multiple interconverting conformers of the cyclic tetrapeptide tentoxin, [*cyclo*-(L-MeAla^1-L-Leu^2-MePhe[(Z)D]^3-Gly^4)], as seen by two-dimensional [^{1}H]NMR. *Biopolymers*, **36** (1995) 135–52.
47. Lange, C., Loutelier, C., Cherton, J-C., Cassier, P., Vey, A. & Pais, M., Desorption of ions from locust tissues. II. Metabolites of E destruxin using negative-ion fast-atom bombardment mass spectrometry. *Rapid Com. in Mass Spectroscopy*, **6** (1992) 28–31.
48. Dumas, C., Robert, P., Pais, M., Vey, A. & Quiot, J-M., Insecticidal and cytotoxic effects of natural and hemisynthetic destruxins. *Comp. Biochem. Physiol.*, **108C** (1994) 195–203.
49. Pinet, E., Gomis, J.-M., Girault, G., Cavelier, F., Verducci, J., Noël, J.-P. & André, F., Tentoxin has at least two binding sites on CF_1 and ε-depleted CF_1 ATPases isolated from spinach chloroplasts. *FEBS Lett.*, **395** (1996) 217–220.